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The studies utilize oxytocin "knock-out" and wild type mice (homozygous for the oxytocin gene) to test the hypothesis that diminished oxytocin and the absence of its effect upon the mammary gland, may in part predispose the breast to neoplastic change and its corollary that exposure to oxytocin may be protective. The technical objectives of this proposal as follows. 1) Determine the prevalence of mammary neoplasia in the progeny of MMTV-infected mice that are bred with OT KO versus WT mice, 2) Determine if there are differences in the susceptibility of MMTV-infected KO versus MMTV-infected WT mice to develop mammary neoplasia when exposed in vivo to ovarian steroid hormones. If oxytocin is shown to be an important factor in breast cancer, exogenous oxytocin may be a potential safe, non-toxic measure in its prevention.

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PI - Signature 7-20-98 Date

TABLE OF CONTENTS

PROGRESS REPORT

Title: Mammary Gland Ontogeny and Neoplasia in Oxytocin Deficient Mice

PI: Janet Amico, M.D.

Introduction: Oxytocin (OT) is a 9 amino acid peptide (M.W. ≈1,000) that is synthesized within the paired paraventricular and supraoptic nuclei (PVN and SON) of the hypothalamus and transported via long axons to the posterior pituitary gland, the site of storage and release of this peptide into the peripheral circulation (1). The primary target tissue of OT's actions is the breast and its primary physiological action is milk ejection during lactation (2). Nursing by the young activates the OT neurons of the hypothalamus, resulting in increased peripheral concentrations of OT (3). OT elicits its biological actions via attaching to its receptor which is located on the myoepithelial cells of the breast (2). The OT receptor (OT-R) is a polypeptide (M.W. ≈42,000) with 7 transmembrane domains and belongs to the class of G protein-coupled receptors (4). When OT attaches to its receptor, the myoepithelial cells contract and milk is expelled. Absence of OT results in deficiency of milk ejection (5).

OT is also released during a variety of reproductive-related events other than nursing, such as late pregnancy and parturition (6,7), breast stimulation (2), and with orgasm during sexual activity (8). Epidemiological studies have shown that many of the conditions that are associated with OT release also appear to confer a relative lower risk of developing breast cancer. Multiparity (9-12), prolonged or repeated lactation (13-17), late menarche (9), and early menopause (18), exert protective effects, whereas nulliparity (9-12), brief or no lactation (13-17), early menarche (9), and late menopause (18) increase the risk of breast cancer. A variety of hormonal influences associated with these events may influence a woman's risk for breast cancer, and OT may be one of those factors.

The absence of an appropriate model has made it difficult to study OT's role in breast neoplasia. The recent development of a mouse in which the gene for OT (5) has been deleted allows us to study the consequences of OT deficiency upon mammary development and neoplasia. The phenotype of this mouse has not yet been extensively characterized but it is known that the animals are fertile and deliver their progeny normally at term, but are unable to nurse their young (5). Although milk production occurs, milk ejection does not (5). These animals have no processed OT and thus are not exposed to OT at any stage of development. Because OT acts upon the breast during events that are considered to lower the relative risk of breast cancer, OT may be protective against mammary neoplasia and its absence may predispose the breast to neoplastic changes.

Preliminary Studies: OT may be important for normal growth and differentiation of mammary cells. The cellular correlates of OT deficiency or over expression in the mammary gland are not yet understood. OT exerts its effects via its receptor. The OT-R is known to activate protein kinase C (PKC) pathways and the transduction of mitogenic signals in cell systems is often linked to protein kinases. OT-R may activate PKC pathways or other mitogenic signaling pathways within mammary cells but this has not yet been investigated.

Work in our laboratory to date includes 1- *in vitro* studies of the OT-R in human breast cancer cell lines; and 2- *in vivo* studies of mammary development and neoplasia in OT deficient mice.

1 - Detection of OT Receptor mRNA by PCR in Human Breast Cancer Cell Lines

To determine the expression of OT-R mRNA in human cancer cells, we examined by RT-PCR the mammary cancer cell lines HS587T (derived from a myoepithelial cancer) and MCF-7 and BT-20 (derived from epithelial cell cancers) as well as non-mammary cancer cell lines K-562 (an erythroleukemia line) and 293 (transformed primary embryonal kidney line). Lines were obtained from the American Type Culture Collection and grown in appropriate media to confluence using methods described below.

Twenty-five cycles of PCR after the reverse transcriptase reaction yielded an amplified band of 397 bp corresponding to a region (1215-1620) of the OT-R mRNA in each mammary cancer cell line but not in the non-mammary cancer cell lines, fig. 1. A GAPDH primer pair was used to be certain that comparable amounts of RNA were used for the reactions. The 397 bp PCR-amplified fragment was also found in pregnant myometrium, which is known to highly express OT-R. The relative amount of the 397 bp RT-PCR product was greater in HS587T cells than MCF-7 cells, when comparable amounts of GAPDH were identified in the samples, fig. 2.

Regulatory studies are being conducted in all three breast cancer cell lines. The results for the HS587T cells are summarized. We investigated the regulation of OT-R in HS587T cells by dexamethasone or OT. Cells were grown using the conditions specified below (see Methods). Cells were grown in serum free medium for 24 hrs prior to a treatment then exposed to dexamethasone or OT for specified times and doses. Cells were harvested, RNA was extracted, and RT-PCR performed upon samples. Values for OT-R are expressed in relation to the amount of GAPDH in the same sample.

Figs. 3 and 4 show the time course and dose response for the 397 bp PCR-amplified fragment during exposure to dexamethasone. Dexamethasone (1μM) increased OT-R levels within 24 hrs of treatment, fig. 3. The response was maximal at a concentration of 1μM, fig. 4. The increase in OT-R by dexamethasone was blocked by treatment of the cells with RU-486 (1μM), a glucocorticoid antagonist, fig. 5.

In contrast, OT (0.1μM) treatment of HS587T cells decreased OT-R expression over the course of 60 min, fig. 6. The concentration necessary for this effect was physiological, 01.μM, fig. 7.

Materials and Methods:

Cell culture and Reagents: Three human breast cancer cell lines were used in the experiments. HS587T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with a 4.5 g/L glucose supplemented with 10% heat inactivated fetal bovine serum (FBS), 10μg/ml of bovine insulin, 100 units/ml of penicillin and 200 units/ml of streptomycin; MCF-7 cells were cultured in MEM medium supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10μg/ml of bovine insulin, 10% FBS and 100 units/ml of penicillin and streptomycin; and BT-20 cells were incubated in RPMI 1640 medium with 10% FBS and the same concentration of antibiotics as the other two cell lines. The cultures were incubated in a 5% CO_2 incubator at 37°C.

In the studies to investigate the regulation of OT-R gene expression by dexamethasone and OT, the experiments were carried out in both serum supplemented

and serum free conditions. When confluence was reached, the cells were trypsinized for 3 min at 37°C, resuspended in the same medium and plated into 6 well plates. Cells were maintained in the media for 2 days. Subconfluent monolayers were washed 3 times with serum-free medium and then incubated with serum-free medium for 24 hr prior to treatment. Cells were treated with serum-free medium plus dexamethasone (Sigma) or OT (Bachem) for specified times and doses. The cells were then harvested for evaluation.

Total RNA extraction. Total RNA was extracted from HS587T, MCF-7 and BT-20 cells by ultrapure Trizol Reagent (Life Technologies). Isolated RNA pellets were suspended in DEPC water followed by a treatment with DNase I FPLC pure, Pharmacia Biotechn, 0.1 unit/1 ug of total RNA). The concentration and purity of total RNA were determined by spectrophotometer at 260nm and 280nm.

Primer design. Two sets of primers were designed and synthesized by Life Technologies. As a target gene, OT-R specific sequences were amplified by using the sense-strand primer 5'CCTTCATCGTGTGCTGGACG 3' nucleotides and antisense-strand primer 5'CTAGGAGCAGAGCACTTATG 3' nucleotides. PCR using this set of primer yields a 397 bp (1215-1602) fragment of OT-R mRNA. The primers have been proven to specifically amplify OT-R mRNA an not genomic DNA. PCR primers used for amplification of GAPDH specific sequences as the "house keeping" gene were as follows: sense-strand 5' GGCTGAGAACGGGAAGCTTG 3' nucleotides and antisense-strand 5' TCTAGACGGCAGGTCAGGTC 3' neuclotides. PCR using this set of primer yields a 510 fragment.

Reverse transcription and PCR amplification (RT-PCR). Total RNA (1 ug) was reverse transcribed into first stand cDNA using 0.5 ug of oligo $_{(dt)}$ 15 primer (Life Technologies) and superscript TM II RNASE reverse transcriptase (100 units/reaction, GIBCO BRL) in a final volume of 20 ul reaction buffer. To ascertain the quality of the RNA preparation, a blank reverse transcription containing all the components except the reverse transcriptase was performed in parallel. Half of the RT-first strand cDNA was used for PCR amplification. To minimize tube to tube variation in RT-PCR, RT and PCR master mix were made separately. PCR amplification was performed with Thermolyne Amplitron II. Samples were denatured at 94°C for denaturation, 1 min at 58°C for primer annealing and 1 min at 72°C for extention/synthesis followed by incubation for 10 min at 72°C. The cycle number was determined with optimal number of cycles that would allow determine of both messengers yet still remain in the log phase of amplification for both OT-R and GAPDH. Following 25 cycles of amplification, an aliquot of PCR fragment was analyzed on 2% agarose gel 0.5 X Tris-Borate EDTA buffer, stained with sybr TM green I nucleic acid gel stain (FM Bio products). The bands were visualized and quantitated using storm 860 blue fluorescence/chemifluorescence of phosphor image system (Molecular Dynamics). OT-R mRNA levels were normalized to GAPDH mRNA.

2- *In vivo* studies of mammary development and neoplasia in OT deficient mice

We have secured heterozygote breeding pairs (6 females and 3 males) and confirmed the genotypes of the animal (OT/-). Successful matings of these animals has

produced F1 progeny of the following genotypes: wild type (OT/OT), heterozygotes (OT/-), and homozygote OT deficient mice (-/-) for study of mammary development and neoplasia. Animals will be sacrificed at various stages of development and the mammary tissue histologically examined for tumors and expression of the OT receptor.

In addition we will determine the prevalence of mammary neoplasia in the progeny of MMTV-infected mice that are bred with the OT deficient mice versus wild type mice. These studies are in progress.

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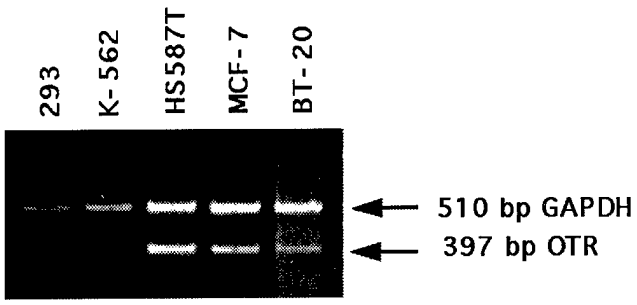

Figure 1. RT-PCR of OT-R. Samples of one microgram of total RNA from HS587T, MCF-7, and BT-20 human breast cancer cells and K-562 erythroleukemia and 293 embryonal kidney cells were reverse transcribed with OT-R and GAPDH specific primers, and each transcript was amplified by PCR. A 397-bp PCR product was detected in the breast cancer cell lines but not the non-mammary cell lines.

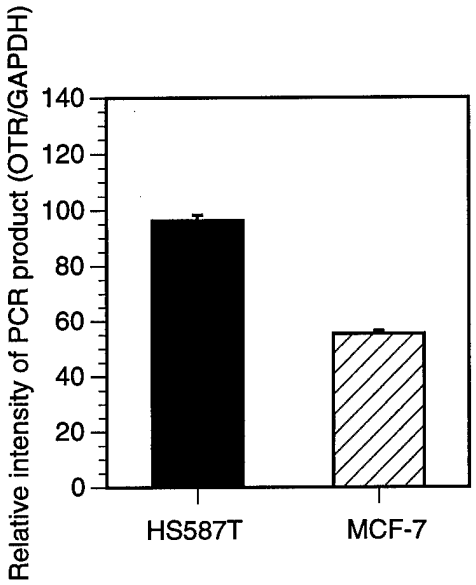

Figure 2. RT-PCR of OT-R in HS587T and MCF-7 cells normalized to GAPDH. The relative amount of OT-R is greater in HS587T cells.

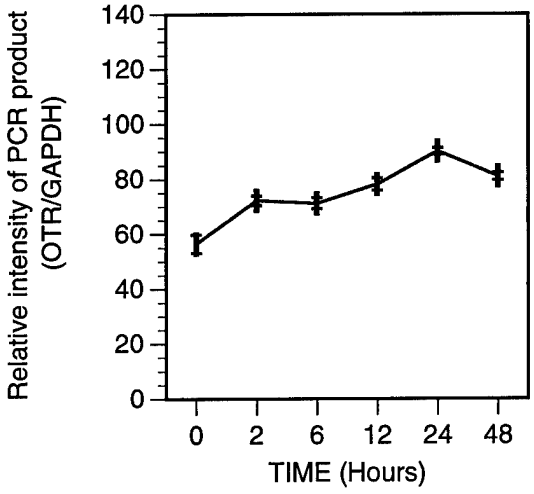

Figure 3. RT-PCR of OT-R in HS587T cells exposed to 1 μM dexamethasone added to serum free medium. Dexamethasone resulted in an increase in the 397-bp PCR product within 24 hrs of exposure, $p<0.01$

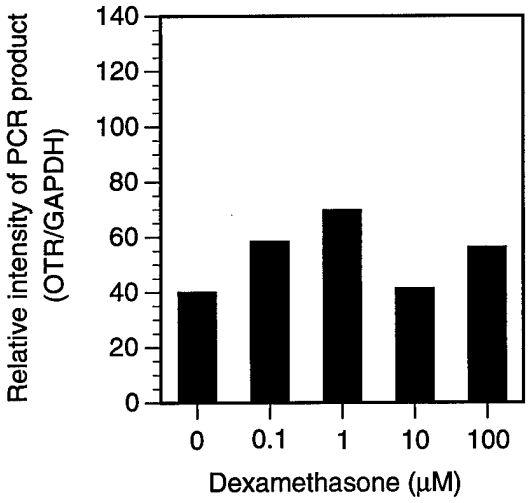

Figure 4. Dose response of the OT-R RT-PCR product treated with dexamethasone in concentrations of 0.1, 1, 10, and 100 μM. The response was maximal at 1 μM.

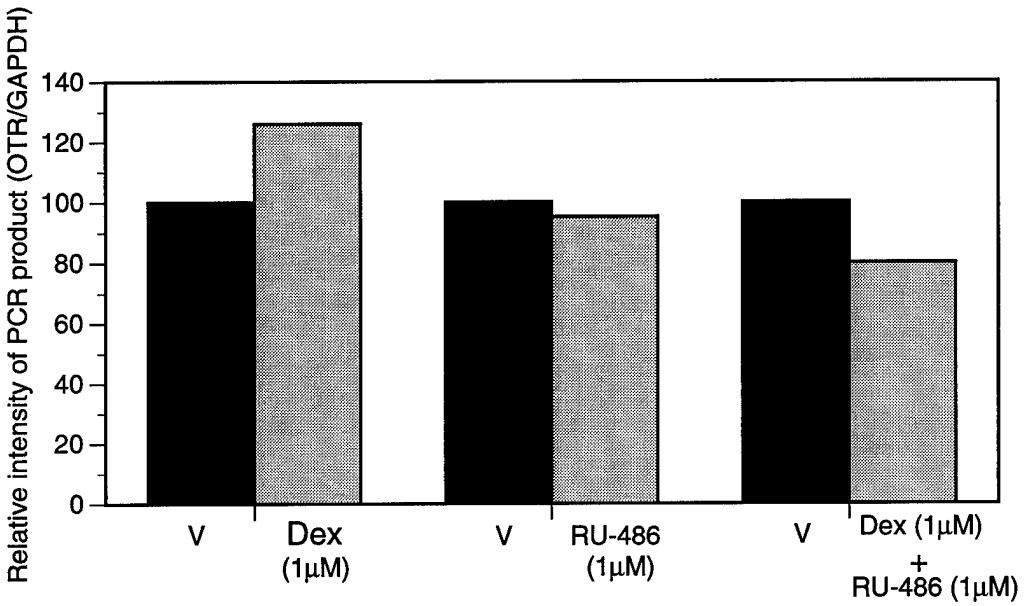

Figure 5. RT-PCR of OT-R in HS587T cells exposed to dexamethasone (1 μM), RU-486 (1μM), or dexamethasone and RU-486, a glucocorticoid antagonist. RU-486 blocked the dexamethasone-induced increase in OT-R. The results are represented as a percent of vehicle (V).

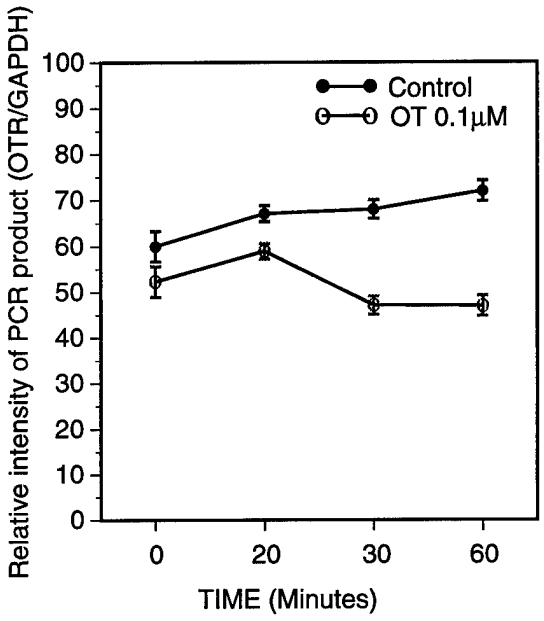

Figure 6. RT-PCR of OT-R in HS587T cells exposed to 0.1 μM OT added to serum free medium. OT resulted in a decrease in the 397-bp PCR product during the 60 min. of exposure.

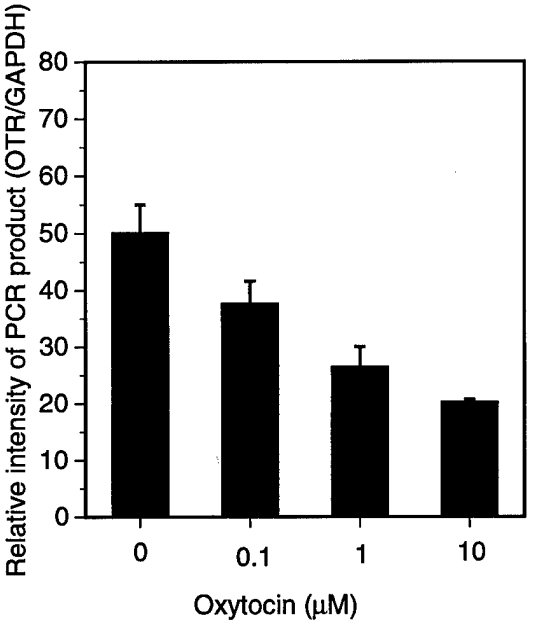

Figure 7. Dose response of the RT-PCR product of the OT-R treated with OT in concentrations of 0.1, 1, and 10 μM. The attenuating effect was seen at each concentration.